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Reliable Propagation of Persian Walnut Varieties Using SSR Marker-based True-to-type Validation

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Abstract. **Simple sequence repeat (SSR) markers were used to authenticate ramets of 11 Persian walnut (*Juglans regia* L.) varieties. All varieties and 28 of their ramets (n = 39) were genotyped with 17 SSR markers. The genetic profiles revealed two off-types: the ramets Serr 4 (S4) and Vina 1 (V1). SSR fingerprints individuating 11 walnut varieties were possible using 13 polymorphic SSRs that could be used in the future to identify clones of these varieties. Except for 'Chandler', each cultivar could be distinguished using a combination of two SSR loci. This result emphasizes the efficacy of the SSR markers in true-to-type validation of walnut orchards.**

Persian walnut (*Juglans regia* L.) is widely cultivated for nut and wood production (McGranahan and Leslie, 2012). Originally cultivated on Persia's plateau, it has been introduced worldwide (Bernard et al. 2018a). In addition to morpho-agronomical characteristics, several biochemical (e.g., enzymes) and molecular markers have been established to identify walnut varieties and to estimate genetic diversity (Bernard et al. 2018b). Identification of varieties based on phenotypic observations is slow, difficult, produces uncertain results due to environmentally induced variability, and may not be possible with juvenile or recently grafted plants (Korir et al. 2013). DNA fingerprinting is commonly used for cultivar and clonal identification in certification programs and in protection of new releases from breeding programs (Foroni et al. 2007) as well as assessment of genetic fidelity of somatic embryogenesis regenerated Persian walnut plantlets (Sadat-Hosseini et al. 2019). Microsatellites are highly reproducible, show high levels of polymorphism, and are based on polymerase chain reaction (PCR) procedures (Korir et al. 2013). DNA fingerprinting by SSRs is therefore a rapid method for true-to-type assessment and variety identification.

To avoid fraud or mistakes in variety/cultivar development and use, walnut breeders can take great advantage from the availability of molecular markers to certify the identities of clonal ramets in walnut orchards by comparison with source plants. Therefore, the aim of this study was to use the simple sequence repeat (SSR) markers to control and validate walnut scions and source plants for reliable propagation.

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Materials and Methods

Plant material. Eleven walnut varieties (Z53, Z63, Lara, Vina, Franquette, Serr, Pedro, Hartley, Z30, K72 and Chandler), growing in the mother orchard of the Horticultural Sciences Research Institute, Karaj, Iran (35°40′N, 51°19′E), were used as the source of scion wood to propagate 28 grafted ramets that were grown in the walnut research orchard of the Department of Horticulture, College of Aburaihan, University of Tehran. Young leaves of mother plants (n = 11) and their clones (n = 28) were sampled from each tree in the spring and stored at –80 °C until DNA extraction and SSR analysis.

DNA extraction. A 100-mg sample of freeze-dried young leaves was ground in Eppendorf tubes with 1800 mL of 65 °C extraction buffer (2% CTAB, 100 mM Tris, 1.4 M NaCl, and 20 mM EDTA, pH 8.3) for 40 min. Then DNA was extracted following the CTAB method (Doyle and Doyle 1990).

DNA concentration was measured by spectrophotometry (Perkin Elmer Co.).

Microsatellite primers and DNA amplification. Seventeen microsatellite loci were amplified: WGA1, WGA4, WGA9, WGA69, WGA89, WGA118, WGA202, WGA225, WGA276, WGA321, WGA331, WGA332, WGA349, and WGA376 (Dangl et al. 2005), and WGA27, WGA32, and WGA71 (Woeste et al. 2002).

PCR reactions were carried out in a final volume of 20 µL containing 60 ng genomic DNA, 2 mM $MgCl_2$, 0.2 mM dNTP, 1× PCR buffer, 1 unit of Taq DNA polymerase (CinnaGen Co., Tehran, Iran), 50 ng/µL of each primer (forward and reverse). PCR was performed in a Bio-Rad Gradient thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA). PCR steps and electrophoresis were according to the Karimi et al. (2010).

Data analysis. The SSR alleles were scored as present (1) or absent (0). Number of alleles, effective number of alleles, observed heterozygosity (Ho), and polymorphism information content were calculated for each SSR locus using PowerMarker V3.2.5 (Liu and Muse 2005). Clustering of the 39 samples was determined using MEGA 4 software.

Results and Discussion

SSR marker analysis. The identification of varieties within and among walnut orchards is important for better management of genetic resources in collections. In this study, a total of 61 alleles were amplified from 17 SSR markers (Table 1). The least polymorphic locus in our study was WGA004 (2 alleles) and the most polymorphic (5 alleles) was WGA276. The average number of alleles per locus was 3.5. The most common allele (91%) was at WGA004 (230 bp). Dangl et al. (2005) studied the genetic diversity of 47 *J. regia* genotypes using 14 SSR primer pairs and identified 73 alleles with an average of 2.5 alleles per locus. Foroni et al. (2007) analyzed 12 SSR loci and reported fewest alleles (N = 3) at WGA001 and WGA118, and the maximum number of alleles (N = 8) at WGA071. In the

Table 1. The loci names, alleles size range, alleles frequency, observed number of alleles (Na), effective number of alleles (Ne), observed heterozygosity (Ho), and polymorphism information content (PIC) for 17 simple sequence repeat loci in 11 *J. regia* varieties.

Locus	Allele size range (bp)	Frequency	Na	Ne	Ho	PIC
WGA001	186–198	0.1–0.47	4	3.03	0.29	0.16
WGA004	230–236	0.08–0.91	2	1.19	0.18	0.08
WGA009	231–246	0.2–0.28	4	3.95	0.72	0.39
WGA027	203–208	0.12–0.74	3	1.70	0.52	0.26
WGA032	166–196	0.1–0.52	4	2.79	0.90	0.43
WGA069	162–182	0.05–0.47	4	2.93	0.54	0.22
WGA071	200–214	0.02–0.48	4	2.79	0.80	0.42
WGA089	212–222	0.02–0.65	4	2.00	0.31	0.16
WGA118	184–199	0.11–0.53	4	2.73	0.62	0.33
WGA202	259–273	0.12–0.43	4	3.19	0.67	0.37
WGA225	189–202	0.26–0.37	3	2.94	0.75	0.42
WGA276	174–211	0.03–0.3	5	3.99	0.67	0.40
WGA321	222–245	0.06–0.53	4	2.65	0.49	0.26
WGA331	273–277	0.16–0.44	3	2.65	0.49	0.26
WGA332	214–225	0.12–0.67	3	1.94	0.42	0.22
WGA349	265–274	0.08–0.56	3	2.24	0.44	0.22
WGA376	242–254	0.06–0.64	3	1.99	0.72	0.40
Mean			3.59	2.63	0.56	0.29

study of Bernard et al. (2018b) among the 217 *J. regia* accessions, the number of alleles per locus ranges from 2 to 17 with an average of 8.92 and the number of effective alleles ranges from 1.18 to 4.61 with an average of 2.84. Level of polymorphism strongly determines how useful an SSR locus will be. A small number of highly polymorphic microsatellite loci can often provide enough information to discriminate among genotypes. Foroni et al. (2005) used six SSR markers to study the Italian walnut landraces in Sorrento. In their study, the total number of observed alleles was 33, with an average of 5.5 alleles per locus.

Observed heterozygosity ranged from 0.18 at locus WGA004 to 0.90 at locus WGA032 (Table 1), and the average Ho was 0.56, a value similar to observed heterozygosity in previous studies on Persian walnut genetic diversity. The average observed Ho reported by Foroni et al. (2005) was 0.68, and the locus with the highest observed Ho was WGA071 (0.81). In the study of Ebrahimi et al. (2011), the average value of observed heterozygosity across loci was 0.72. The per-locus observed Ho ranged from 0.10 to 0.68 with a mean value of 0.47 in the study of Bernard et al. (2018b).

In this study, new alleles were described for the Iranian varieties at the following markers: WGA001 (198 bp) for Z63 and K72; WGA071 (214 bp) for Z30; and WGA276 (211 bp) for Z53 and Z30. The most informative markers were WGA001, WGA009, WGA069, and WGA202, whereas WGA004, WGA027, WGA032, and WGA376 did not have any polymorphism useful for discriminating among these samples. Except for Chandler, each cultivar could be distinguished using a combination of only two SSR loci. Thirteen SSR markers were powerful enough to produce a unique DNA fingerprint for each of the 11 varieties, and thus they could be used to certify cultivar identity with respect to accessions in this study.

Cluster analysis. Cluster analyses revealed well-separated groups (Fig. 1). All ramets of the same source genotypes were placed with their source clones except Vina1 and Serr4. The reasons for misclassification may have included mislabeling of samples, failed grafts, and collecting the wrong scion wood from the mother orchard. This kind of mistake usually happens when grafters do not use right labeling protocols and/or do not use different painting colors to characterize each cultivar. Additionally, it is possible that the scion samples were not taken from the original source of the varieties but were obtained from other sources in the Horticultural Sciences Research Institute collection, where their propagation using asexual methods such as in vitro micropropagation and grafting, may have resulted in off-type (mutation).

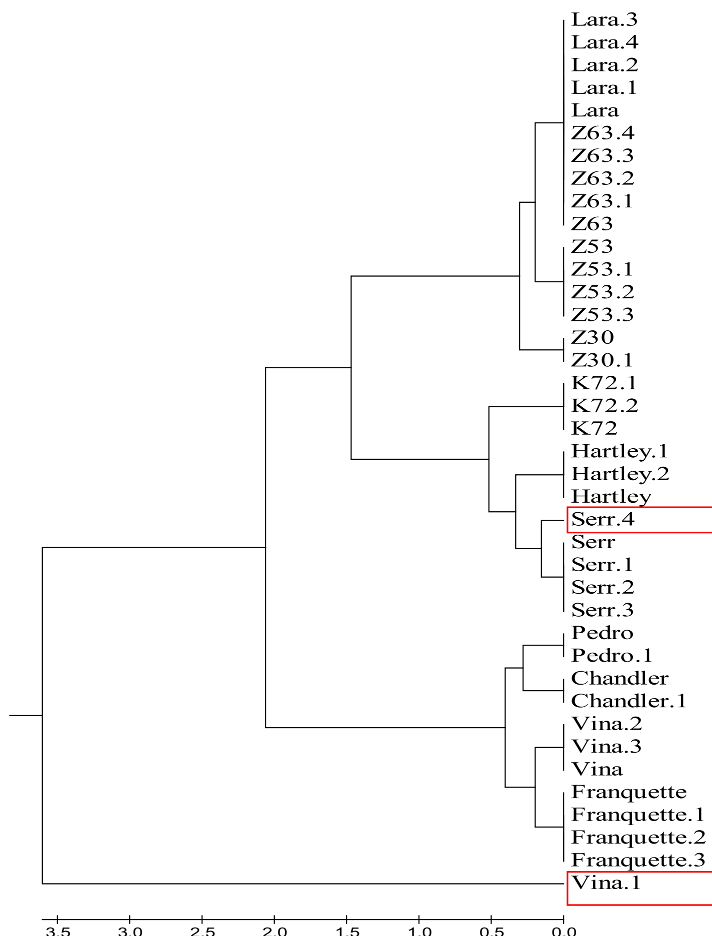

Fig. 1. Cluster analysis of 39 varieties of walnut using simple sequence repeat markers based on unweighted pair group method with arithmetic mean analysis and Jacard's similarity coefficient. Vina. 1 and Serr. 4 (indicated by the red box) are two failures in grafting or off-types, ramets.

Conclusion

Genetic validation of trueness-to-type and identity with source material is essential to the nursery trade, which needs simple and low-cost methods to certify stock and prevent errors in propagation. This study confirmed the SSR markers as an efficient tool in detecting errors and validating Persian walnut (*Juglans regia*) varieties. More important, our results show the suitability of SSR marker-based true-to-type tests in the validation of walnut grafted ramets and genotype identification. Application of SSRs for trueness-to-type test is of great value in breeding programs for reducing propagation errors and thus facilitating breeding effort and progress.

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